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by

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A Kinetic Study of the Leuchs Anhydrides in Aqueous Solution. I.*

by Paul D. Bartlett and Richard H. Jones

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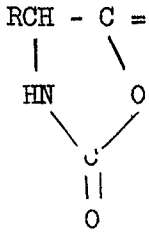
Abstract

The reaction of an anhydro-N-carboxy-amino acid (Leuchs anhydride) with water is subject to general basic catalysis and is not catalyzed by acids. The reaction with the amino group of an amino acid anion is so rapid that it can compete successfully with hydrolysis. The reactions of anhydro-N-carboxyglycine, anhydro-N-carboxy-d and l-alanine and anhydro-N-carboxy-α-aminoisobutyric acid with water and some amino acids have been followed kinetically by carbon dioxide evolution and their rate constants determined (Tables 2 and 3). The possibility of controlled peptide synthesis in aqueous solution from the Leuchs anhydrides is discussed and experiments are reported showing the limitations of such a procedure.

Introduction

The anhydrides (I) of N-carboxy-α-amino-acids, first prepared by Leuchs,[1]



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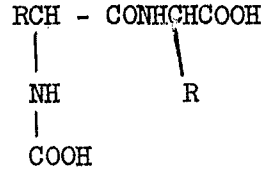

II

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1. H. Leuchs, Ber. 39, 857 (1906)

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have been investigated chiefly in inert solvents where with controlled amounts of active-hydrogen compounds as initiators, they lead to polypeptides[2] of moderate or high molecular weight

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2. For a review see E. Katchalski, Advances in Protein Chemistry, Volume 6, Academic Press New York, 1951, pp. 123-185. More recent work: D. G. H. Ballard and C. H. Bamford Symposium on Peptide Chemistry, Special Publication No. 2, Chemical Society, London, 1955, pp. 25-48; E. R. Blout and R. H. Karlson, This Journal, 78, 941 (1956). P. Doty and R. D. Lundberg, ibid., 00, 0000 (1956).

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while at very low temperatures the decarboxylation of the initial carbamic acid (II) can be repressed sufficiently by salt formation[3] to afford good yields of simple peptides.

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3. J. Leggett Bailey, J. Chem. Soc. 1950, 3461

Even more attractive possibilities of control of this reaction would present themselves if it were possible to operate in aqueous solutions; with this in mind, we have studied the nature and rates of the reactions of some simple N-carboxyanhydrides in water.

Reactions of N-Carboxy-Anhydrides with Water - The N-carboxy-anhydrides of glycine, dl-alanine and α-amino-isobutyric acid, on solution in water at 0°, evolve carbon dioxide at a rate convenient for measurement.

The reactions were followed by observing the increase in pressure in a closed, initially evacuated system; with the first and last of these anhydrides a rate measurement by following volume increase at constant pressure agreed with the results of the pressure method. Except for a short initial period (see below) the reactions, as followed in this manner, were of the first order, yielding rate constants as follows:

Table 1

Rate Constants for Uncatalyzed Hydrolysis at 0°C

N-Carboxy-Anhydride of	No. of runs	Concentration Range	k_1 sec.$^{-1}$ x 10^3	Mean Deviation
Glycine	6	.01-.028	4.1	0.23
dl-Alanine	3	.022-.028	6.4	0.23
α.Aminoisobutyric Acid	5	.019-.028	2.4	0.12

Reactions of anhydro-N-carboxy-α-aminoisobutyric acid in 0.1 M barium chloride, 0.1 M hydrochloric acid, and 1.0 M sulfuric acid yielded the same average rate constant, 2.4×10^{-3} sec.$^{-1}$, as the reaction in pure water, with a mean deviation of $\pm$ 0.07×10^{-3}. The effect of acid catalysis upon the hydrolysis of the anhydride is therefore negligible.

Carbon dioxide is not evolved when the carboxy-anhydrides react with sodium or barium hydroxide. An attempt was made to measure the rate of the reaction of anhydro-N-carboxyglycine with hydroxyl ion by following the conductivity of a solution of barium hydroxide to which the anhydride was added. There was a very rapid reaction which produced 74% of the expected change in conductivity within the first forty seconds, followed by a slower process to which a second-order rate constant of 0.30 L./mole sec. could be assigned. The latter process is far too slow to be the reaction of anhydride with hydroxyl ion, as a later experiment proves. According to[4]

Stadie and O'Brien,

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4. W. C. Stadie and H. O'Brien, J. Biol. Chem. 103, 521 (1933)
112, 723 (1935)

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carbamates such as the Siegfried salts come rapidly to equilibrium with carbon dioxide and amino acid anion, but the carbon dioxide is much more slowly equilibrated with bicarbonate and carbonate. The calculated equilibrium concentration of carbonate ion during the preparation of a Siegfried barium salt is more than sufficient to precipitate barium carbonate; the non-appearance of such a precipitate is evidently the result of slow attainment of the carbonate equilibrium. The slow reaction observed in the conductivity experiments may well be the conversion of hydroxyl ion into carbonate.

Hydrolysis of N-Carboxy-Anhydrides in Buffer Solutions

During this work it became apparent that the amino acid formed by hydrolysis of the anhydride was itself competing appreciably with water, especially in the case of glycine, for reaction with the anhydride after the reaction was under way. The effect of this competition could be reduced by operating either in excess base to prevent decarboxylation of N-carboxy-glycine, or in a buffer solution sufficiently acid to keep the amino group of the amino acid overwhelmingly in the ammonium ion form. An acetate buffer was adopted containing 1.00 M sodium acetate and 0.875 M acetic acid, and having a pH of 4.76. The experiments described above indicated that the hydrolysis of an N-carboxy anhydride is subject to basic but not to acid catalysis. In view of the possibility of general basic catalysis (e.g. catalysis by acetate ions as well as by hydroxyl ions), a series of rate determinations were made in which anhydro-N-carboxyglycine was hydrolyzed at 0° at concentrations from 0.023 to 0.026 M in this acetate buffer at various buffer dilutions up to tenfold, the ionic strength being maintained constant at 1.00 by means of potassium chloride. The results fit

the equation

$$k = 0.00028 + 0.00049 \left[OAc^-\right] \qquad \text{(time in sec.)}$$

closely. That this is catalysis by the acetate ion and not by acetic acid is already indicated by the complete insensitivity of the rate to strong acids in the case of anhydro-N-carboxy-α-aminoisobutyric acid. From this result it appears that about 30% of the rate measured in pure water was due to the overlaying of the hydrolysis by a reaction with amino acid formed during the hydrolysis and existing at a varying pH during the reaction, which did not produce a clear deviation from first-order kinetics. Later work verified this indication that the carboxy-anhydrides do indeed react preferentially with amino groups even in aqueous solution.

Kinetic Salt Effect - The salt effect on the hydrolysis of anhydro-N-carboxyglycine is negative, as shown by the first-order rate constants in buffer solutions consisting of 0.1 M sodium acetate and 0.0875 M acetic acid alone ($k = 0.00043$ sec.$^{-1}$). No study was made to apportion this between "primary" and "secondary" kinetic salt effect.

The "Induction Period" - Most of the manometric runs had an initial period of ten minutes during which the reaction appeared to be very slow, before the uniform first-order rate of gas evolution was attained. Since the carbon dioxide is the product of the second of two successive reactions,

$$I \longrightarrow \underset{\displaystyle NHCOOH}{\overset{\displaystyle |}{RCHCOOH}} \longrightarrow \underset{\displaystyle NH_2}{\overset{\displaystyle |}{RCHCOOH}} + CO_2$$

we considered the possibility that the decarboxylation of the carbamic acid was a slow enough reaction to account for this effect. This was tested by taking advantage of the fact that a stable salt of glycine N-carboxylic acid can be prepared[5] by saturating a solution of barium glycinate with carbon dioxide.

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5. M. Siegfried, Z. Physiol. Chem., 44, 85 (1905)

At 0°, in the absence of excess barium hydroxide, this saturation was accomplished without any precipitation of barium carbonate. The decarboxylation of N-carboxyglycine was now observed by the rapid addition to the stable Siegfried salt of enough acetic acid to make an acetate buffer of pH 4.76, or in other experiments to give an unbuffered solution of pH 0-2. The evolution of carbon dioxide in these experiments followed an approximately first-order course with k_1 varying from 0.023 to 0.042 sec.$^{-1}$. However, when a solution of sodium carbonate was acidified in the same manner, similar results were obtained, k_1 being again from 0.023 to 0.042 sec.$^{-1}$. That the measurable reaction was just the rate of evolution of carbon dioxide from aqueous solution was verified in a series of experiments in which the rate constants varied from 0.005 to 0.030 sec.$^{-1}$ as the speed of the magnetic stirrer in the apparatus was varied over an approximately fivefold range. We conclude that the slow approach to linearity in the kinetic curves is due to the appreciable time required for the establishment of liquid-vapor equilibrium, a steady state being approached as the carbon dioxide evolved in a period of a few minutes becomes a smaller fraction of the total present.

The rate of equilibration of carbonic acid with water and carbon dioxide, an important factor in physiological processes, is greater than the rate of gas evolution here measured. The rate constant is reported[6] as 1.97 sec.$^{-1}$ by Roughton and coworkers and 1.4 sec.$^{-1}$

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6. F. J. W. Roughton, R. Brinksman and R. Margaria Phil. Trans. Roy. Soc. A 232 65 (1933) W. C. Stadie and H. O'Brien, J. Biol. Chem., 103, 521 (1933)

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by Stadie and O'Brien at 0°. The rate constant for decarboxylation of unsubstituted carbamic acid at this temperature is reported[7] to be even greater, 80 sec.$^{-1}$.

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7. F. J. W. Roughton, This Journal, 63, 2934 (1941)

It appears that whatever the position of the equilibrium in the carbonation of glycine, this equilibrium is attained rapidly in comparison with the reactions whose rates are being measured here.

Reaction of N-Carboxy-Anhydrides with Amino Acids - With the information at hand it was possible to measure the rates of reaction of the N-carboxyanhydrides with various amino acids in aqueous systems. For this purpose it was not possible to suppress the hydrolysis of the anhydride entirely, but the rate constant of this process could be accurately evaluated and subtracted from the overall pseudo-unimolecular rate constant observed in the presence of an excess of amino acid in the same buffer solution. The reactivity of an amino acid toward an N-carboxyanhydride resides entirely in the free amino group and the fraction of the amino acid molecules possessing such a free amino group is controlled by the buffer solution. By knowing the second acid dissociation constant of an amino acid and the pH of the buffer, it should therefore be possible to determine the rate constant for reaction of an N-carboxyanhydride with the free-base form of any amino acid. In the 0.1 M acetate buffer of pH 4.74 the pseudo-unimolecular rate constant k_{obs} was used as follows to determine the second-order rate constant k_{AA} for the reaction of anhydro-N-carboxyglycine with amino acid:

$$k_{obs} = 0.00043 + k_{AA} \text{ (Amino Acid)}$$

and for anhydro-N-carboxyalanine:

$$k_{obs} = 0.00071 + k_{AA} \text{ (Amino Acid)}$$

In the latter case the correction to be made for hydrolysis rate amounted to as much as 80% of the quantity being measured; in most cases the relative rates

were more favorable than this. An idea of the degree of reliability of the results may be had from the data in Table 2, which were determined in order to learn how great the selectivity might be between the optical isomers of alanine in reaction with d- or l-anhydro-N-carboxyalanine.

Table 2

Optically Active Anhydro-N-Carboxy-Alanines and Optically Active Alanines in Acetate Buffer at 0°. 0.100 M NaOAc, 0.0875 M HOAc

Anhydride Concentration	Alanine Concentration	k_1 sec.$^{-1}$ x 10^3	$\frac{k_1 - .00071}{(\text{alanine})}$ L./mole sec. x 10^3
L-Alanine Anhydride Alone			
.0396	0	0.70	
.0426	0	0.71	
.0465	0	0.70	
	Average =	0.70	
D-Alanine Anhydride Alone			
0.454	0	0.71	
0.423	0	0.72	
0.446	0	0.72	
	Average =	0.72	
L-Alanine Anhydride + L-Alanine			
.0408	.225	1.02	1.29
.0416	.449	1.31	1.33
.0442	.449	1.31	1.33
			Average = 1.32
D-Alanine Anhydride + D-Alanine			
.0454	.337	1.15	1.30
.0439	.449	1.31	1.33
			Average = 1.32
D-Alanine Anhydride + L-Alanine			
.0456	.225	0.96	1.12
.0438	.337	1.07	1.06
.0422	.449	1.04	1.10
			Average = 1.09

Table 2 (Continued)

Anhydride Concentration	Alanine Concentration	k_1 sec.$^{-1}$ x 10^3	$\frac{k_1 - .00071}{(\text{Alanine})}$ L./mole sec. x 10^3
L-Alanine + D-Alanine			
.0420	.225	0.96	1.13
.0438	.449	1.16	1.00
.0411	.449	1.19	1.07
.0449	.337	1.07	1.07
		Average =	1.10

The four possible pairs were run in triplicate, with mean deviations of 1-3% in the second-order rate constants determined. There was agreement between the values for d-anhydride with d-alanine and for l-anhydride with l-alanine, and also between the constant for d-anhydride with l-alanine and that for l-anhydride with d-alanine. In each case the reaction between the configurationally similar reactants was about 20% faster than that between the configurationally opposite reactants. It is not surprising that the difference is small, since neither reacting center is itself asymmetric.

Table 3 summarizes the second-order rate constants for reaction of anhydro-N-carboxy-glycine and alanine with several amino acids and peptides. These measurements are made under conditions where only a minute fraction of the amino acid exists with its amino group in the free-base form, yet even under these conditions the amino acid competes successfully with the water for reaction with the N-carboxy-anhydride. It is of interest to calculate how fast the active form of the amino acid (having its amino group free) is reacting with the anhydride. For this purpose there are two molecular species to be considered, the anion $H_2NCHRCOO^-$, and the unfavored uncharged form $H_2NCHRCOOH$. The fraction of the former species present in our buffer solution is $10^{(4.74 - pK_2)}$ and the fraction of the latter species is 10^{pK_z}, where K_2 is the second ionization constant of the

amino acid and K_z is the equilibrium constant between the dipolar ion and the[8] uncharged amino acid.

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8. J. T. Edsall and M. H. Blanchard, This Journal, 55, 2344 (1933)

The latter quantity has been evaluated for most of the amino acids with which we are concerned by Edsall and Blanchard. If for glycine we extrapolate their value of pK_z from 25° to 0° by means of their value of ΔH = 11,500 cal., we find that pK_z under our conditions is -5.79 and this leads to an estimate of the fraction of neutral glycine present at pH 4.74 as 1/1,500,000. From the values of pK_2 at 0° tabulated in Table 3 the fraction of anion has been estimated; for glycine it is 1/620,000. Thus about two-thirds of the free amino group which is responsible for the reaction with N-carboxy-anhydride at pH 4.74 is situated on the glycine anion, and the other third is on neutral glycine. These are by no means accurate figures in view of the assumptions underlying the estimate of k_z, but they suggest that we should not be too far wrong in calculating the bimolecular rate constant for reaction between N-carboxyanhydride and glycine anion if we were to attribute all the reaction observed at pH 4.74 to this species. In favor of this approximation is the expectation that a negative charge on the carboxyl group might enhance the nucleophilic reactivity of the amino group, so that the anion would be inherently more reactive than the neutral form. Against this is the possibility that an undissociated carboxyl group in the amino acid might be strategically located to assist catalytically in the reaction of its amino group with the carbonyl group in the anhydride, which might enhance the importance of the rare neutral species in contributing to the observed rate. In a series of experiments in acetate buffers at constant acetate concentration and ionic strength but varying acetic acid concentration and pH, the rate of reaction was inversely

proportional to the acetic acid concentration.

Table 3

Anhydro-N-Carboxyglycine and Amino Acids at 0° in Acetate Buffer (0.1 M Sodium Acetate, 0.0875 M Acetic Acid)

Amino Acid	k_{AA}, L/mole sec. x 10^3	$pK_2^{0°}$	$(k_{AA})^-$ corr.
Glycine	3.8 ± 0.25	10.53[a]	2330
dl-Alanine	1.10 ± 0.12	10.62[a]	830
α-Aminoisobutyric acid	0.265 ± 0.045	10.57[a]	179
l-Leucine	1.85 ± 0.13	10.73[a]	1800
Glycylglycine	23.7 ± 0.17	8.86[b]	311
Triglycine	29.0 ± 0.17	(8.58)[c]	200
	Anhydro-N-Carboxy-d-Alanine		
dl-Alanine	1.2	10.62[a]	917
dl-Alanyl alanine	4.4 ± 0.7	(9.17)[c]	118
l-Leucine	1.11	10.73[a]	1100

a. From an equation of H. S. Harned and R. A. Robinson, Trans. Faraday Soc., 36, 973 (1940) and data selected by J. T. Edsall in Cohn and Edsall, Amino Acids, Peptides and Proteins, New York, (1943) p.79.

b. J. Greenstein, J. Biol. Chem., 101, 603 (1933).

c. Estimated assuming the same temperature coefficient as for the amino acids, from data in Cohn and Edsall, p.84.

This provides evidence that the amino acid anion is the dominant species in the reaction with anhydro-N-carboxyglycine, rather than the neutral form which is present at constant concentration in this series of buffers.

Accordingly, in Table 3 the corrected bimolecular rate constant for reaction of anhydride with each amino acid anion has been computed by multiplying k_{AA} by the factor $10^{(pK_2 - 4.74)}$. It will be seen that these rate constants are very large, indicating a surprising ability of the amino group to compete with solvent water. More will be said of this in a later section.

The rate constants for reaction of anhydro-N-carboxyglycine with glycine, glycylglycine and glycylglycylglycine are in the ratio of 1:6:7.5. This means

experimentally that in the acetate buffer there is a strong tendency toward the formation of higher peptides through competition of the glycylglycine already formed during a run with the glycine added, and so on for the higher peptides. As the last two columns show, this is not due to an inherently higher reactivity of the peptide anions toward the anhydride in comparison with glycine, but rather to the fact that the second ionization of di- and triglycine occurs at less basic pH values and there is more anion in proportion present at pH 4.74. The corrected rate constants, referred to the anions, are in the reverse order: 12:1.6:1. Likewise, anhydro-N-carboxyalanine reacts more than three times as fast with alanylalanine at pH 4.74 as with alanine, although the anion of the latter is the more reactive by a factor of almost eight.

A regularity is discernible in the anion rate constants when a "Brönsted plot" is made of the logarithms of the rate constants referred to pure anion against the logarithms of the second ionization constants, the measure of the thermodynamic basicity of the amino groups. The table contains data for three compounds with amino groups on primary carbon atoms reacting with anhydro-N-carboxyglycine and for three compounds with amino groups on secondary carbon atoms reacting with anhydro-N-carboxyalanine. Each class of amino compound determined a straight line (Fig. 1), the rates for the secondary amino acids and peptides being slower than for comparably basic primary compounds. In the case of the one amino acid examined which has an amino group on a tertiary carbon atom, the point if plotted would lie below either line. Toward alanine and leucine the reactivities of anhydro-N-carboxyglycine and the corresponding alanine derivative do not appear to be very different. Also plotted in Fig. 1 are the rate constants for three reactions in the "secondary" class, from Part II (see following paper)

The Possibility of Controlled Peptide Syntheses in Aqueous Solution - The selective reaction of anhydro-N-carboxyglycine with amino acids in aqueous solution raises the question whether it would be possible to control this process with enough precision

to synthesize a peptide step by step by the successive addition of a series of N-carboxyanhydrides to a growing peptide in solution in water, without isolating the peptide at each step. Obviously this would require very high yields in each step in order to be feasible for chains of any length. Our measurements show that there are two competing reactions of importance to interfere with the clean production of a desired peptide. One of these is the reaction of the anhydride with hydroxyl ion, the other is the reaction of the product peptide with anhydride to give the peptide of one more amino acid unit. The rate of uncatalyzed or buffer-catalyzed hydrolysis can be negligible in the pH range above 7.

As far as the reaction with hydroxyl ion is concerned, we did not succeed in measuring its rate. We can make an estimate of the second-order rate constant for reaction of anhydro-N-carboxyglycine with hydroxyl ion by extrapolating the Brönsted plot of Figure 1, which shows reactivity toward the anhydride as a function of basic strength, to pK 14, finding thus a rate constant of 2×10^5 L. per mole second. Since we know the rate constants for uncatalyzed hydrolysis and for reaction of the anhydride with glycine as a function of pH, we can make a hypothetical calculation to the effect that

$$\text{Rate of hydrolysis} = [A]\left(.00041 + k_{OH}10^{pH-14}\right)$$

$$\text{Rate of diglycine formation} = \frac{2330\,[A][G]}{1 + 10^{10.53-pH}}$$

By differentiating to make the ratio of the second rate to the first a maximum, we find that the pH should be most favorable around 8, at which point diglycine formation should be favored over hydrolysis by a factor of 34 times the glycine concentration. This factor is not very sensitive to pH in this region, being adversely affected by the constant water hydrolysis in the more acid media, and by the fact that the hydroxyl ion concentration continues rising beyond pH 10-11

where the concentration of the reactive form of the amino acid ceases to increase. Thus if we could operate in solutions of glycine, averaging 1 M in concentration during a run, 97% yields of diglycine should be available.

The competition between glycine and the desired product, glycylglycine, for reaction with the N-carboxyanhydride is more serious. We have observed that because of the lower basicity of glycylglycine (pK_2 8.86) in comparison to glycine (pK_2 10.53) the dipeptide appears to be the stronger competitor toward the anhydride when examined in the buffer of pH 4.75. This situation should be reversed at pH 10-11, where both amino compounds are largely in their reactive forms, but the ratio between the rates of formation of diglycine and triglycine can never exceed 2330/311 = 7.5, times the ratio of glycine and diglycine concentrations present. (Table 3). For this reaction to be useful, it would be necessary that we start with equivalent amounts of glycine (or other desired starting material) and N-carboxyanhydride. The concentration of diglycine in this example would start at zero and would approach a concentration whose ratio to that of glycine was equal to k_G/k_{GG}, the ratio of the two rate constants. At the time of total consumption of the anhydride, an amount of tripeptide would have been formed which was equal to that of the glycine remaining unattacked, in this case 12% of the total, hydrolysis being neglected. For higher peptides, where the differences between members of the series become less, the outlook is correspondingly less encouraging.

The experiments on peptide synthesis in water bear out these conclusions in general. Quantitative experiments were made in which, in each of a series of solutions of different pH, equimolar amounts of glycine and anhydro-N-carboxyglycine were introduced and the reaction was allowed to run to completion. The remaining glycine was then determined by the Van Slyke ninhydrin technique, by which amino acid can be estimated in the presence of peptides which do not evolve carbon

dioxide on treatment with the reagent. Table 4 lists the results obtained.

Table 4

Attempts at Synthesis of Dipeptides from Anhydro-N-Carboxyglycine and Glycine at 0°

Solution	pH[a]	% Polymer with Anhydride Alone	Final Conc Glycine[b] M x 10^2	% Dipeptide (Side Reaction → Triglycine)	% Dipeptide (Side Reaction → Glycine)
0.174 N $Ba(OH)_2$	(13.2)	11	5.5	0	31
0.50 M Na_2CO_3	11.51	18	5.4	0	33
1.0 M NaH_2BO_3 + .2 M H_3BO_3	10.89	31	.65	68	91
0.5 M NaH_2BO_3	10.61	50	.35	83	96
0.5 M Na_2CO_3 0.5 M $NaHCO_3$	(10.3)	10	> 5.5	0	31
Catalytic amount $Ba(OH)_2$	(9.5)	98	3.0[c]	0	63

a. pH values are those of the solution before the addition of anhydride or amino acids. Values in parentheses are calculated. Others were measured at 28° using a Beckman pH meter with glass electrode for solutions of pH 10.

b. Initial concentrations of all solutions: 4.0×10^{-2} M glycine and 4×10^{-2} M N-carboxyglycine anhydride.

c. Calculated from a reaction where two portions of glycine anhydride were used in an attempt to synthesize triglycine.

In a reaction yielding only hydrolysis and dipeptide formation there will be two molecules of amino acid remaining in excess for each anhydride molecule hydrolyzed; for this reaction not only wastes anhydride, but adds to the store of amino acid. In a reaction yielding only di- and tripeptide, the amount of tripeptide will be equal to the amount of amino acid remaining unattacked. In Table 4 the yield of the desired dipeptide has been computed on both assumptions, and recorded in the last two columns.

Two solutions in which the pH was controlled by borate buffers at pH's of 10.89 and 10.61 gave results in the expected range. The above considerations having shown that competition from tripeptide formation is more important in this pH range than loss by hydrolysis, the figures of 68 and 83% are the most likely ones for the yields of dipeptide. Solutions where similar pH's were maintained by means of carbonate buffers showed large amounts of glycine remaining at the end of the run. The significant difference may well be the presence of carbon dioxide at sufficient concentration to convert an appreciable fraction of the glycine into the related N-carboxylate. This would lower the overall ability of the glycine to compete for the anhydride, relative to hydroxyl ion. We do not know the position of the carbonation equilibrium for glycylglycine, but in accord with its lower basicity it is possible that this dipeptide should be less carbonated and hence the carbonate buffer might well affect the relative rates of formation of di- and tripeptide as well as the relative rates of reaction with glycine and hydroxyl ion. This explanation is borne out by some experiments in which anhydro-N-carboxyglycine was allowed to react directly in the buffer solutions without added glycine. Column 3 of Table 4 shows that the ability of the glycine formed by hydrolysis to react further with the anhydride is much reduced in the carbonate buffers relative to the buffers containing borate.

The two experiments using barium hydroxide, in catalytic amounts and in excess, gave results as expected from the above kinetic discussion. With the small amount of base the pH began at 9.5 and dropped. Polymerization was so important that only 2% of amino acid remained in the control experiment without added glycine. Obviously here the formation of tri- and higher peptides is the important side reaction and the yield of dipeptide from the 1:1 reaction mixture is zero, as indicated in column 5. In 0.174 N barium hydroxide, anhydride alone yields 89% of glycine. Hydrolysis is therefore rapid, as expected, and the results from the 1:1

experiment mean a 31% yield of diglycine. The upshot of these experiments is that the conditions for selective formation of glycylglycine are rather critical, and the selectivity is not sufficient to make this a useful general method for the stepwise synthesis of polypeptides.

Experimental

Reagents - Various methods were used for preparing the Leuchs anhydrides but by far the most satisfacory was the method[9] of Ben-Ishai and Katchalski

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9. E. Ben-Ishai and E. Katchalski, J. Am. Chem. Soc. 74, 3688 (1952)

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using the N-carbobenzoxy amino acids and phosphorus tribromide. The purity of the anhydrides was best determined by the quantitative measurement of the carbon dioxide liberated. This was done during each kinetic run and consistent values were obtained from the various samples of anhydrides used. The anhydrides were stored in a desiccator over phosphorus pentoxide, being stable under these conditions for several weeks. In practice, however, the anhydrides were used within three days of the final recrystallization.

Benzyl chloroformate was obtained from three sources. It was prepared[10] by the method of Organic Syntheses,

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10. Organic Syntheses 23, 13

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some was purchased from the Mann Research Laboratories, and some was kindly donated by Dr. E. R. Blout.

N-carbobenzoxyglycine was prepared from benzyl chloroformate and glycine by the method of Organic Syntheses.

N-carbomethoxyglycine was prepared by the method described[11] by Schramm

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11. C. H. Schramm, Thesis, Harvard University, 1947

N-carbethoxyglycine was prepared from ethyl chloroformate and glycine in a procedure similar to that used for N-carbomethoxyglycine; the melting point of the crude material was 74-78°.

Anhydro-N-carboxyglycine was prepared by the method of Katchalski. The crude product was recrystallized four or five times from dry ethyl acetate. Good yields of the anhydride were also obtained with 20.9 gms. of N-carbobenzoxyglycine and 25 g. of phosphorus pentachloride dissolved in 150 ml. of dry ethyl acetate at 0°. The mixture was shaken every two or three minutes and the temperature maintained at 0° for one-half hour. The solution was allowed to warm to room temperature and was filtered to remove excess phosphorus pentachloride. The ethyl acetate was removed under vacuum at temperatures up to 60° and more ethyl acetate was added. After the removal of most of the ethyl acetate, the residue was recrystallized from ethyl acetate.

N-carbomethoxy-α-amino-isobutyric acid was prepared by the method of Schramm.

Anhydro-N-carboxy-α-amino-isobutyric acid was made from N-carbomethoxy-α-amino-isobutyric acid and thionyl chloride, according to the method of Schramm. The product was recrystallized six times from a benzene-cyclohexane mixture.

N-carbomethoxy-dl-alanine, melting point 75-77°, was prepared according to the method of Schramm.

N-carbobenzoxy-dl-alanine, N-carbobenzoxy-d-alanine and N-carbobenzoxy-l-alanine were prepared in 90% yield by the Organic Syntheses procedure, described for N-carbobenzoxyglycine. The product was recrystallized by dissolving in chloroform and adding petroleum ether. The melting points were 111-115°, 85.2-86.0° and 85.0-85.5° respectively.

Anhydro-N-carboxy-dl-alanine was prepared from N-carbobenzoxy dl-alanine and

phosphorus pentachloride, following Schramm's directions for the preparation using N-carbomethoxy-dl-alanine. The product was recrystallized several times from dry benzene, although it was necessary to seed each time. Several attempted preparations from the N-carbomethoxy amino acid were unsuccessful, as was the attempted use of thionyl chloride or phosphorus tribromide on the N-carbobenzoxy-dl-alanine.

Anhydro-N-carboxy-d-alanine and anhydro-N-carboxy-l-alanine were easily prepared by the method of Katchalski from the corresponding N-carbobenzoxyalanine and phosphorus tribromide. The product was recrystallized three or more times from dry benzene. It was occasionally necessary to add dry petroleum ether to cause crystallization of the anhydrides.

Benzene, ether and petroleum ether (30-60°) were reagent grade dried over sodium wire.

Cyclohexane was Eastman Kodak's technical grade distilled and dried over sodium wire.

Reagent quality ethyl acetate was dried over calcium hydride and distilled.

Glycine and dl-alanine were obtained from the Eastman Kodak Company and were twice recrystallized from ethyl alcohol-water solution, before being used in kinetic runs. These and all the other amino acids and peptides were dried at 110° before use.

α-amino-isobutyric acid and l-leucine were obtained from Eastman Kodak Company and were recrystallized once from an ethyl alcohol-water solution before being used in kinetic runs.

d-Alanine was obtained from Schwartz Laboratories and was used without further purification. $\alpha_D^{25} = -14.8$ (C=2 in 2N hydrochloric acid).

l-Alanine was obtained from Schwartz Laboratories. $\alpha_D^{25} + 14.3°$ (C=2 in 2N hydrochloric acid).

Glycylglycine was obtained from the Nutritional Biochemical Corporation and was recrystallized from 50% ethyl alcohol before use.

Glycylglycylglycine was obtained from the Nutritional Biochemical Corporation and was recrystallized from water.

dl-Alanyl-dl-alanine was obtained from the Nutritional Biochemical Corporation and was used without further purification.

Kinetic Procedure - The reaction vessel was a 250 ml. Erlenmeyer flask equipped with a side arm to which a mercury manometer could be attached through a $^{10}/_{30}$ joint. The mercury manometer was made of 1 mm. capillary tubing with a reservoir made of 20 mm. tubing, so that the change in height in the reservoir would be negligible during the course of a run. Evacuation was accomplished through a stopcock attached to the arm between the flask and the manometer. The Erlenmeyer flask was closed with a $^{24}/_{40}$ cap equipped with a glass hook from which a glass bucket containing the anhydro-N-carboxy-amino acid could be suspended by a platinum wire.

First the weighed amino acid or peptide, when any was used, was added to the Erlenmeyer flask, followed by the water or buffered solution (50 ml. for the runs with optically active anhydro-N-carboxy-alanine and 100 ml. in all other cases). The system was assembled using silicone stopcock grease and the reaction flask was immersed in an ice bath for one hour before the start of the run. The ice bath, stirred by air and surrounded by two concentric beakers, was so arranged as to permit magnetic stirring through the bottom by means of an Arthur H. Thomas Company magnetic stirrer and a plastic coated magnet within the reaction flask.

After the attainment of thermal equilibrium, a weighed amount of the anhydride was suspended from the cap and the system was evacuated. After three or four minutes, the anhydride was dropped into the solution by agitation of the reaction vessel and the run was begun. The manometer was always tapped immediately before reading and its response characteristics were determined before use. The pressure at infinite time

was that observed after at least ten times the half life.

In studying the decomposition of the Siegfried salts, a solution of amino acid in barium hydroxide was placed in the reaction flask and carbon dioxide was bubbled through the solution until added phenolphthalein turned colorless. In this instance, acetic acid or sulfuric acid was suspended in the glass bucket and was dropped into the basic solution at zero time to start the reaction.

For the kinetic experiments using the volume method, the manometer was replaced by a connection to a 50 ml. gas burette and a butyl phthalate manometer. The connection was made almost completely from glass with two short joints of tygon tubing.

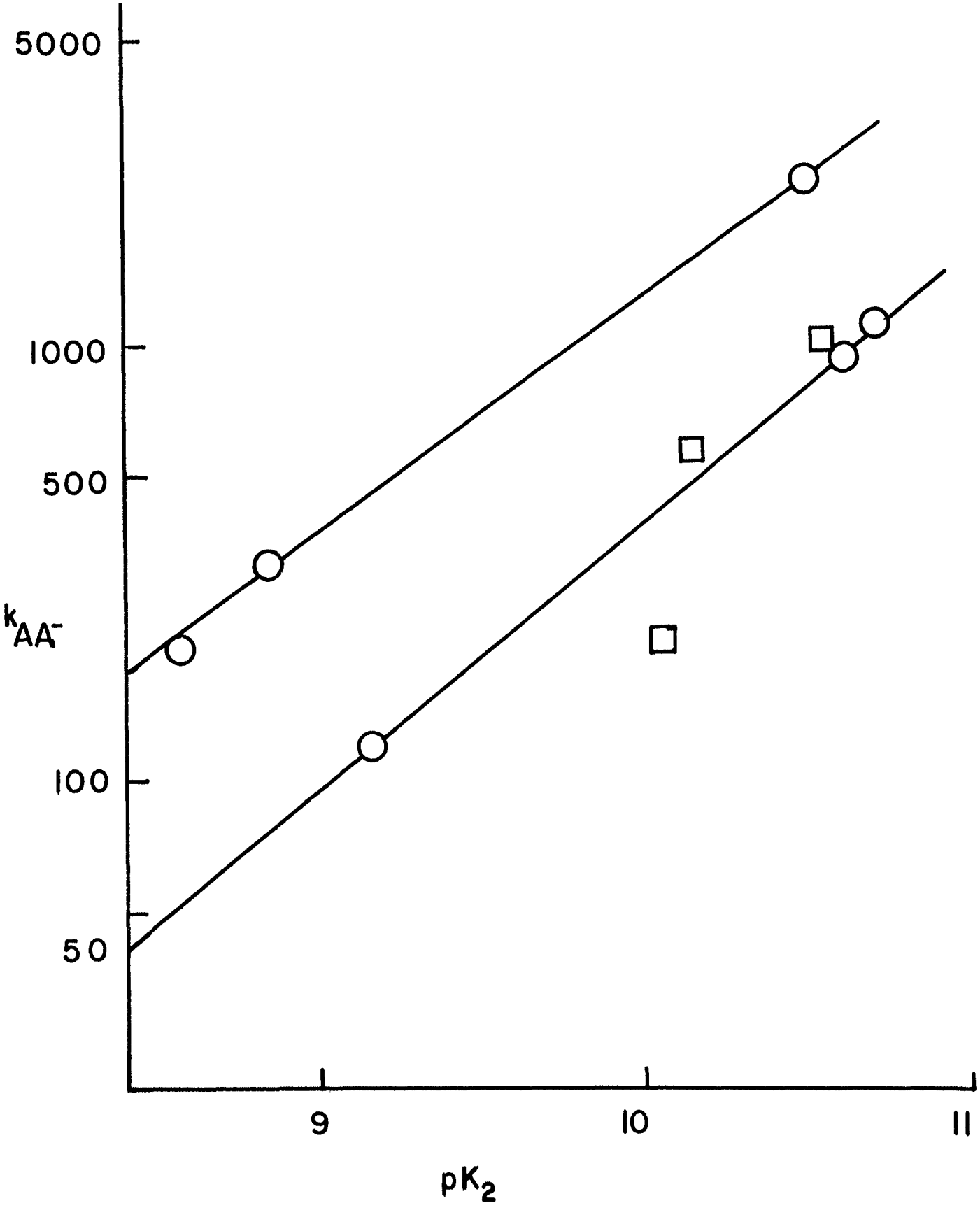
The conductivity cell used in the reactions between anhydride and barium hydroxide had platinized electrodes, 1 cm. square and 1 cm. apart. The resistance of the cell was measured with a conductivity bridge, model RC-1B made by Industrial Instruments Inc. The runs were started by adding barium hydroxide solution at 0° to a solution of anhydro-N-carboxyglycine in water at 0°.

Product Studies - Exactly 5 ml. of 0.400 molar glycine and 50 ml. of an appropriate buffer were placed in a stoppered 250 ml. Erlenmeyer flask and cooled to 0°. Exactly 0.202 g. (.002 mol.) of anhydro-N-carboxy-glycine was added and the mixture was rapidly stirred with a magnetic stirrer. After several hours the solution was neutralized to pH 7.0 (brom-thymol blue) with dilute hydrochloric acid and quantitatively transferred to a 250 ml. volumetric flask. The solution was acidified with 50 ml. of pH 4.5 acetate buffer (1 M) and diluted with water to 250 ml. A sample of the solution (5 ml.) was analyzed by the Van Slyke ninhydrin method. Acetate buffer was used instead of citrate buffers since all the kinetic runs that were analyzed were already buffered with acetate. The procedure used did not give quantitative yields of carbon dioxide, but was helpful as a qualitative measure of the amino acid concentration. Thus standard alanine solutions yielded 98 $\pm$ 1% of the calculated carbon dioxide,

whereas standard glycine solutions yielded 92 ± 3%. Glycylglycylglycine, when analyzed by this method, gave .074 mcls. of glycine per mol. of glycylglycylglycine.

Cambridge, Massachusetts.

Figure 1 - Logarithmic relationship between k_{AA^-}, the second-order rate constant for reaction in water at 0° between an anhydro-N-carboxy-amino-acid and an amino acid or peptide anion, and pK_2, the second ionization constant of the amino acid. Upper line - anhydro-N-carboxyglycine with triglycine, glycylglycine, and glycine anions. Lower line - anhydro-N-carboxyalanine with alanylalanine, alanine, and leucine. See Table 3. Square points - data from Part II, This Journal, 00, 0000 (1957): phenylalanine, methionine, aspartic acid.


5000
1000
500
100
50
k_{AA^-}
9
10
11
pK_2

(Contribution from the Converse Memorial Laboratory of Harvard University)

A Kinetic Study of the Leuchs Anhydrides in Aqueous Solution. II[1]

by Paul D. Bartlett and Donald C. Dittmer

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Abstract

Rate constants are reported for the reaction in water at 0° between anhydro-N-carboxyalanine and phenylalanine, methionine, aspartic acid, histidine, cysteine and glutathione. Cysteine reacts 45 times as fast as methionine. In the reaction of cysteine with anhydro-N-carboxy-dl-phenylalanine, the concentration of free SH groups shows an initial decline followed by a return to its original value. This is interpreted as an initial reaction of the anhydride with the thiol group followed by an intramolecular transfer of the phenylalanyl group from sulfur to nitrogen. Glutathione shows a similar rapid reaction, but the thiol titre drops much more and is slow to return, as would be expected from the less favorable location of groups for an intramolecular acylation. The effect of conditions upon the reaction of alanine with both anhydro-N-carboxy-dl-alanine and anhydro-N-carboxy-dl-phenylalanine in aqueous solution has been studied.

It was shown in Part I[2]

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2. P. D. Bartlett and R. H. Jones, This Journal, 00, 0000 (1957)

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that the anhydro-N-carboxy derivatives of certain amino acids reacted in aqueous solution with amino groups so rapidly as to raise the hope that this might be a useful method for controlled, stepwise peptide synthesis. In a further exploration of systems of this kind, we have measured the rate constants listed in Table 1 between anhydro-N-carboxy-dl-alanine and amino acids of different types. The rate constants were determined as described in Part I[2]. An attempt has been made in Table 1, as in the cases previously reported, to refer the rate constants to

Table 1

Reactions of Anhydro-N-Carboxy-dl-Alanine at 0° in Acetate Buffer (0.102 M NaOAc, 0.0875 M HOAc) at pH 4.75

Added compound	k_1, sec.$^{-1}$ x 10^3	k_{AA} L./mole sec. x 10^3	pK^c_2	k_{AA}- L./mole sec.
None	0.715			
dl-Phenylalanine	0.772	1.0	10.06	200[d]
dl-Methionine	0.988	2.3	10.14	565[d]
dl-Aspartic Acid	0.837	1.4	10.53	840[d]
l-Cysteine	14.8[a]	103	9.11	1770
l-Histidine	6.25	61[b]	10.10	1370
Glutathione	2.83	68	9.59	4640

a. Did not show clean first-order kinetics
b. pH increased during run
c. E. J. Cohn and J. T. Edsall, Proteins, Amino Acids and Peptides, Reinhold Publishing Corporation, New York, 1943, p.84 ff., assuming same temperature coefficient as for glycine and alanine.
d. Plotted in Figure 1, Part I, This Journal, 00, 0000 (1957).

the active anion of the amino acid by means of the published second ionization constants of the amino acids in question.

Table 1 shows that cysteine and glutathione react substantially faster with anhydro-N-carboxy-alanine than does the closely comparable methionine which lacks the sulfhydryl group. Some experiments were carried out in which the sulfhydryl group was titrated with iodine during the reaction between cysteine and anhydro-N-carboxy-dl-phenylalanine. The results are shown in Figure 1, together with the results of a similar run on the tripeptide glutathione. In both cases there is an initial attack of the carboxy-anhydride on the sulfhydryl group. In the case of cysteine, the sulfhydryl titre had dropped at the end of one minute to 67% of that initially present, but had already begun to rise again. The rapid formation of thioester is evidently followed up immediately by a transfer of the acyl group from sulfur to nitrogen with the formation of a normal peptide. Such transfer reactions have been observed before[3], being a consequence of the reactivity of thioesters

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3. T. Wieland, E. Bokelmann, L. Bauer, H. U. Lang and H. Lau, Ann., 583, 129 (1953).

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and the fact that such a transfer can take place here by way of a six-membered cyclic transition state.

In the case of glutathione the sulfhydryl titre also drops but does not return nearly so rapidly. At the end of an hour the sulfhydryl titre has reached 9% of that initially present and appears to be still dropping. After 20 hours the titre is again 9%; after three days it has risen again to 45%. The difference between the cases of cysteine and glutathione would appear to be largely the less favorable situation in the latter case for the transfer of an alanyl group from sulfur to nitrogen internally.

The attempts reported in Part I to achieve high yields in the formation of specific peptides were extended to the use of anhydro-N-carboxy-dl-alanine and anhydro-N-carboxy-dl-phenylalanine. As judged by the amount of amino acid remaining after reaction, the yields obtained from these anhydrides and alanine were never as high as the best yields of glycylglycine under comparable conditions. Experiments were conducted at pH 9, 10, 11 and 12, the pH being generally controlled by the use of a Beckman auto-titrator. With anhydro-N-carboxy -alanine as with anhydro-N-carboxy-glycine the highest yields of dipeptide were obtained at pH 10, but these yields never reached 90%. The presence or absence of a borate or phosphate buffer and the use of lithium, sodium or thallium hydroxide as the titrating base produced no marked effect upon the course of the reaction. In the case of anhydro-N-carboxy-dl-phenylalanine the yields were consistently below 80% and the sensitivity to pH appeared to be somewhat less. These results are all consistent with the view that the aqueous reactions of the anhydro-N-carboxy- amino acids are determined by a set of protonation and carboxylation equilibria which are established rapidly compared to the speed of the attack of the water or amino acids upon the anhydride.

Experimental

Materials - dl-Alanine, dl-methionine and dl-aspartic acid were Eastman White Label grades. dl-Phenylalanine was the CP grade obtained from H. M. Co., Ltd. l-Cysteine, glutathione and l-histidine were from the Nutritional Biochemicals Corporation. Benzyl chloroformate was from Mann Biochemicals. N-carbobenzoxy-dl-alanine and N-carbobenzoxy-dl-phenylalanine were prepared by the method of Organic Syntheses[4].

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4. H. Carter, R. Frank and H. Johnston, Organic Syntheses, 23, 13 (1943).

Anhydro-N-carboxy-dl-alanine and anhydro-N-carboxy-dl-phenylalanine were prepared from the corresponding N-carbobenzoxy derivatives and phosphorus tribromide by the method of Ben-Ishai and Katchalski[5].

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5. E. Ben-Ishai and E. Katchalski. This Journal 74, 688 (1952)

They were recrystallized from ethyl acetate and petroleum ether. Ethyl acetate was Merck reagent grade dried over calcium hydride.

The manometric kinetic determinations were carried out as described in Part I.

The reactions of l-cysteine and glutathione with anhydro-N-carboxy-dl-phenylalanine were done in an acetic acid-sodium acetate buffer (pH 4.66) at 0°. The anhydride was added in 10 ml. of dimethylformamide. Aliquots were taken at various times, quenched in 2 N hydrochloric acid and cooled in an ice-bath. A measured amount of standard iodine solution was added to react with the excess SH groups and the excess of iodine was back titrated with standard sodium thiosulfate solution using starch as an indicator. There was no reaction between cysteine and dimethylformamide under the reaction conditions employed. The accuracy of the glutathione reactions was impaired by precipitation early in the reaction.

The solution of the product of reaction between l-cysteine and anhydro-N-carboxy-dl-phenylalanine was oxidized with hydrogen peroxide. The resulting material was submitted to paper chromatography with butanol-acetic acid[6] resulting in three ninhydrin-positive spots. Two of these were readily identified

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6. K. Slotta and J. Primosigh, Nature 168, 696 (1951)

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as dl-phenylalanine and cysteic acid. From a large number of paper chromatograms carried out on small aliquots of the solution the area of the unknown spot was cut out and eluted. The solution of the unknown was evaporated and the α-amino group of the peptide was removed with nitrosyl chloride[7]

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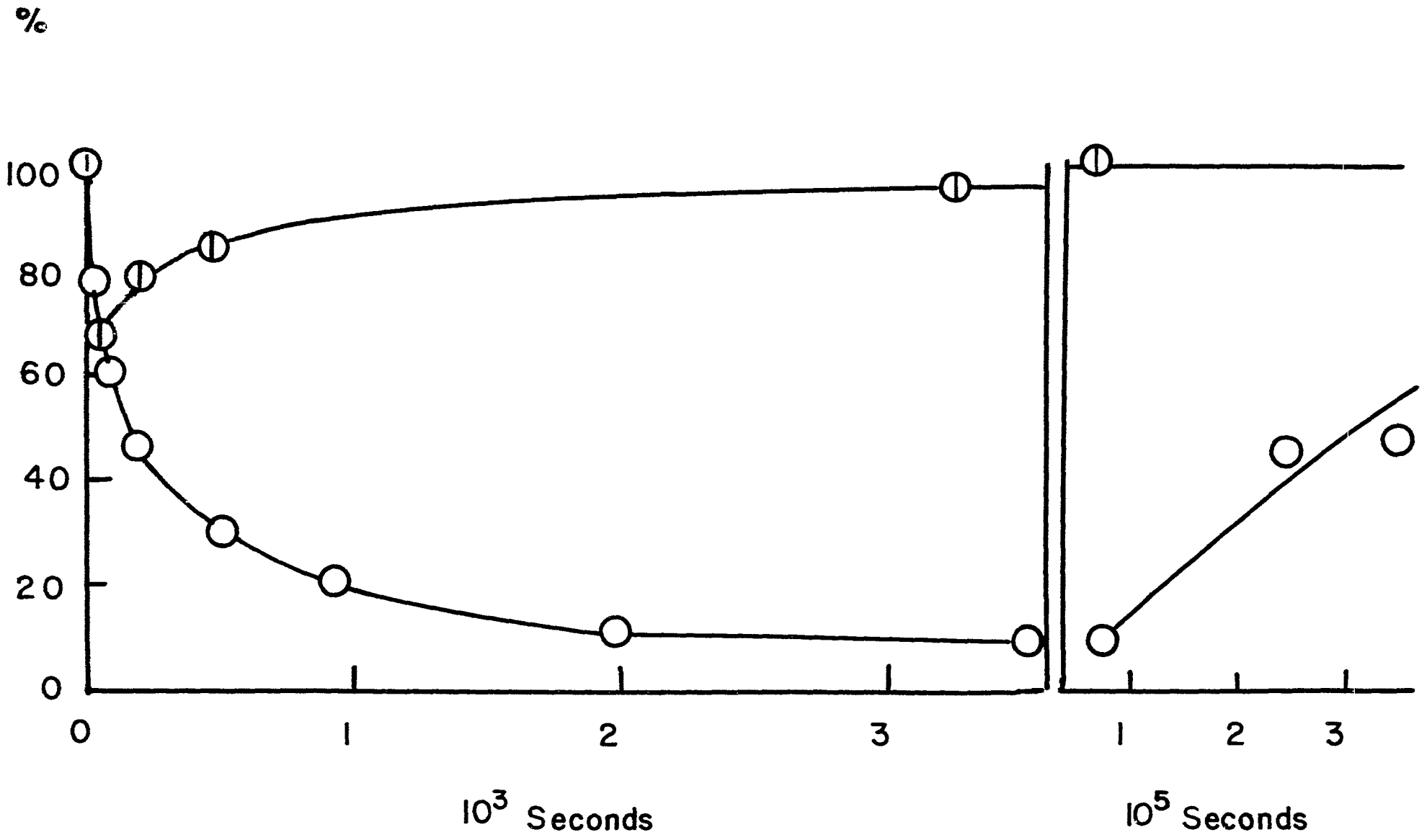
7. R. Consden, A. Gordon and A. Martin, Biochemical Journal 41, 590 (1947)
F. Sanger and H. Tuppy, Biochemical Journal 49, 463 (1951)

The product was then hydrolyzed and chromatographed on paper. One ninhydrin-positive spot appeared which had the same R_f value as cysteic acid. The original dipeptide was accordingly probably dl-phenylalanyl-cysteine.

Cambridge, Massachusetts.

Figure 1 - Decline and return of sulfhydryl titre during reaction of anhydro-N-carboxy-dl-phenylalanine with cysteine (dashed circles) and with glutathione (open circles). Note break in time scale.


%
100
80
60
40
20
0
0
1
2
3
1
2
3
10^3 Seconds
10^5 Seconds